Title: Progenitor cell markers predict outcome of patients with Hepatocellular Carcinoma beyond Milan criteria undergoing liver transplantation

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List of abbreviations

HCC: Hepatocellular carcinoma

LT: Liver transplantation

CK19: Cytokeratin protein 19

HR: Hazard Ratio:

EpCAM: Epithelial cell adhesion molecule

OS: Overall survival

miVI: microvascular invasion

TGFb: Transforming growth factor beta

CT: Computed Tomography

MRI: Magnetic Resonance Imaging

AFP: Alpha-fetoprotein

IHC, immunohistochemical

RPS6: ribosomal protein S6

FDR: False discovery rate

H&E: Hematoxylin and eosin

PPV: Positive predictive value

NPV: Negative predictive value

HCV: Hepatitis C virus

HBV: Hepatitis B Virus

VIF: Variance Inflation Factor

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ABSTRACT

Background and Aims: In patients with hepatocellular carcinoma (HCC), liver transplantation (LT) is an excellent therapy if tumor characteristics are within the Milan criteria. We aimed to define genomic features enabling to identify HCC patients beyond Milan criteria who have acceptable transplant outcomes.

Methods: Among 770 consecutive HCC patients transplanted between 1990 and 2013, 132 had tumors exceeding Milan criteria on pathology and were enrolled in the study; 44% of the patients satisfied the 'up-to-7 rule' [7=sum of the size of the largest tumor and the number of tumors]. Explant tumors were assessed for genomic signatures and immunohistochemical markers associated with poor outcome.

Results: At a median follow-up of 88 months, 64 patients had died and 45 recurred; the 5-year overall survival (OS) and recurrence rates were 57% and 35%, respectively. Cytokeratin 19 (CK19) gene signature was independently associated with recurrence [Hazard ratio (HR)=2.95, p<0.001], along with tumor size (HR=3.37, p=0.023) and presence of satellites (HR=2.98, p=0.001). S2 subclass signature was independently associated with poor OS (HR=3.18, p=0.001), along with tumor size (HR=5.06, p<0.001) and up-to-7 rule (HR=2.50, p=0.002). Using the presence of progenitor cells markers (either CK19 or S2 signatures) patients were classified into poor-prognosis (n=58; 5-year recurrence 53%, survival 45%) and good-prognosis (n=74; 5-year recurrence 19%, survival 67%) (HR=3.16, p<0.001 for recurrence, and HR=1.72, p=0.04 for OS).

Conclusions:

HCC patients transplanted beyond Milan criteria without gene signatures of progenitor markers (CK19 and S2) achieved survival rates similar as those within Milan criteria.

Once prospectively validated, these markers may support a limited expansion of LT indications.

INTRODUCTION

Liver transplantation (LT) is an effective treatment option for hepatocellular carcinoma (HCC) when disease is defined by the widely accepted Milan criteria [1, 2]. Transplantation for patients within the Milan criteria generally yields a 5-year overall survival (OS) of 70% and a recurrence rate less than 15% [1-3]. Several efforts have been made to expand the criteria based on tumor size and number [4-8]. Although downstaging of tumors beyond Milan criteria is accepted by some UNOS regions, it has not yet been adopted by international consensus guidelines of LT [9] or guidelines of management of HCC [2]. Mazzaferro et al. proposed the up-to-7 rule [the sum of the number of tumor nodule(s) and the maximum diameter of the nodule(s) must not exceed the value of 7] which, in the absence of microvascular invasion (miVI) results in 5-year OS above 70% [8]. This study along with previously published data confirmed miVI to be a key predictor of recurrence in patients with HCC [10]. However, this information cannot be used in pre-transplant decision-making as an indication of transplantation since miVI is only diagnosed based on post-surgical histological assessment. This highlights the limitations of the current image-based prognostic algorithm for selecting HCC patients for LT.

Genome-wide transcriptome profiling has identified several key deregulated genes, molecular pathways and signatures associated with disease progression and prognosis in HCC [11, 12]. Activation of specific molecular pathways such as transforming growth factor-beta (TGFb) as well as presence of progenitor cell markers such as cytokeratin 19 (CK19) and epithelial cell adhesion molecule (EPCAM) have been associated with more aggressive biological tumor characteristics and rapid disease spread [13-35].

In a previous study, we identified gene signatures that significantly improved prediction of HCC recurrence after surgical resection [21]. Here we sought to define if gene signatures are also able to identify patients with HCC beyond Milan criteria who nevertheless may have acceptable outcomes with LT. In addition, we are providing the transcriptomic landscape of patients at more advanced states of the disease compared with those undergoing resection or transplantation according to Milan criteria, as per AASLD/EASL guidelines [2, 36].

MATERIALS AND METHODS

Patient cohorts and tissue samples

A total of 770 patients were transplanted for HCC between 1990 and 2013 at Mount Sinai Hospital, New York (n=590) and Mayo Clinic, Scottsdale-Arizona (n=180). Among these, we selected 132 patients (Mount Sinai: n=94, Mayo: n=38) with HCC beyond Milan criteria by pathological assessment, for whom base-line and follow-up clinical information and archived fixed tissue of viable tumor were available **(Figure 1).** The study protocol was approved by the respective Institutional Review Boards. See Supplementary Methods.

Genome-wide transcriptome profiling

Total RNA was subjected to transcriptome profiling using Whole-Genome DASL-HT (Illumina). See Supplementary Methods. GEO accession number: GSE62743.

Immunohistochemistry

Immunohistochemistry was performed for the evaluation of nuclear β-catenin as a surrogate for Wnt pathway activation; phosphorylation of ribosomal protein S6 (RPS6) as the major downstream effector of activated AKT-mTOR pathway; CK19 and EPCAM as surrogate of progenitor cell origin. See Supplementary Methods.

Bioinformatics and statistical analysis

Presence of previously reported HCC gene signatures (**Supplementary Table 1**) was evaluated (**Figure 2**). See Supplementary Methods.

RESULTS

Patient characteristics

Clinico-pathological data and outcomes of the patients are summarized in **Table 1**. Most of the patients were male (80%) and hepatitis C virus (HCV)-infected (71%). The median size of the largest tumor was 4 cm (range 1-9 cm). Tumors exceeded Milan criteria due to single tumor larger than 5 cm (5%), 2 to 3 tumors with the largest tumor larger than 3 cm (39%), or more than 3 tumors within the liver (57%). Satellite lesions (defined as HCC nodules < 2 cm in diameter within 2 cm from main tumor [37]) were present in 18% and miVI was present in 84% of the patients. Serum alpha fetoprotein (AFP) was > 200 mg/dL in 17% of cases. At the end of follow-up, 64 patients had died, OS at 5- and 10-years was 57% and 42%, respectively. HCC recurrence developed in 45 patients, 5 and 10-year cumulative recurrence rates were 35% and 44%, respectively (**Figure 3**).

Clinical variables identified as predictors of recurrence on multivariate analysis included tumor diameter >5 cm [p=0.023, Hazard Ratio (HR) = 3.37] and the presence of satellites (p<0.001, HR=2.98). Tumor diameter >5 cm (p<0.001, HR=5.06) and outside up-to-7 rule (HR=2.50, p=0.002) predicted poor survival (**Tables 2 and 3)**.

Prevalence of prognostic liver cancer gene signatures

Among the 22 liver cancer gene signatures tested, 16 were able to sort patients into good and poor prognosis groups with a FDR<0.05 (**Figure 2A**). The human [21] and rat [28] CK19 gene signatures were present in 40% and 42% of patients, respectively. Signatures associated with poor clinical outcome and/or more aggressive biological features were generally present in the same set of tumors. At least one poor-prognosis signature was present in 84 patients (64%), this being a higher rate than we have

previously reported in patients treated with resection [21]. Twenty-five tumors (19%) harbored more than 10 poor prognosis/aggressiveness gene signatures. VI signature was positive in 22% of cases, 93% of which were positive for histological miVI. The majority of tumors lacking poor prognosis/aggressive signatures were enriched by the S3 subclass signature (39/48, 81%), which is associated with smaller, higher proportion of well-differentiated tumors [13].

The gene signatures analyzed were consistent with 3 distinct clusters or major molecular classes of HCC **(Figure 2B)**: (1) *Proliferation and cell cycle activation* (Cluster A [16], Cholangiocarcinoma [29], Proliferation [20], Recurrence [24], S1 [13], TGFb [23], MET [18], and G3 [19] signatures), 2) *Proliferation-progenitor cell origin* (Hepatoblastoma C2 [26], Class S2 [13], EpCAM [25] and VI [22] signatures), which includes a subclass of CK*19* positive tumors (CK19 human [21] and rat [28] signatures), and 3) *non-proliferative class*, enriched by tumors positive for CTNNB1[20] or class S3 [13, 33]. Interestingly, there was no association between the underlying etiology [HCV, Hepatitis B Virus (HBV) or alcohol] and the expression of signatures.

Comparing the classification of signatures between this cohort and previously published results from resection cases [21], we noticed a higher frequency of tumors positive for $\geq$ 6 signatures of poor prognosis (32.6% vs 25%, respectively). Similarly, our cohort presented a higher prevalence of CK19 human signatures (41% vs 34%, respectively), suggesting that it includes more advanced tumors compared with our previous report on resected patients [21].

Immunohistochemical (IHC) analysis

CK19 immunostaining was positive in 6 tumors (5%), and was significantly associated with the presence of the human (p=0.03) and rat (p=0.04) CK19 gene signatures, as well as VI signature [22] (p<0.01) (**Supplementary Table 2**). EPCAM immunostaining was positive in 20 tumors (15%), and was significantly associated with EPCAM signature (p=0.01), human and rat CK19 signatures (p=0.01 and p=0.02, respectively), Proliferation subclass signature (p=0.01), and VI signature (p=0.01) (**Supplementary Table 2**). Nuclear staining of β-catenin was observed in 27 tumors (21%), and was positively associated with CTNNB1 class signature (p=0.01) (**Supplementary Figure 1, Supplementary Table 2**). In addition, 12 samples (9%) had overexpression of cytoplasmic β catenin with one third of these in CTNNB1 class (4/12, 33%). Phospho-RPS6 staining was positive in 75 tumors (57%) with no obvious correlation with any of the evaluated gene signatures.

Prognostic relevance of liver cancer gene signatures in LT treated patients beyond Milan

Prognostic gene signatures in HCC were initially developed in patients undergoing resection, a treatment indicated for early stages of the disease. Univariate Cox regression analysis demonstrated that VI [22], G3 subclass [19], and S2 subclass [13] signatures were associated with overall survival, along with patient's age, tumor size, and up-to-7 rule (**Table 2**). In the multivariate analysis only S2 signature [13] (HR=3.18, p=0.001), tumor diameter >5 cm (HR=5.06, p<0.001), and outside up-to-7 rule (HR=2.50, p=0.002) were independent predictors of survival. VI [22], G3 [19], Proliferation [20], CK19 human [21], CK19 rat [28], Cholangiocarcinoma [29], Cluster A [16] and S3 [13] gene signatures were associated with HCC recurrence in univariate

analysis along with tumor size, AFP and satellites from clinical variables as well as RPS6 from IHC (**Table 3**). In multivariate analysis, CK19 human [21] (HR=2.91, p=0.001), tumor diameter > 5 cm (HR=3.37, p=0.023) and satellites (HR=2.98, p<0.001) were independent predictors of recurrence.

Of note, histological miVI predicted neither survival nor recurrence, presumably because in this cohort it was present in the large majority of cases (112 patients, 84.8%). Also, IHC positivity for reported progenitor cell markers also failed to improve outcome prediction.

Combination of progenitor cell signatures (CK19 and S2) discriminates prognostic subgroups in transplant patients

Multivariate analyses demonstrated prognostic significance of two progenitor-related gene signatures, CK19 [21] and S2 [13]. Furthermore, S2 subclass signature was also able to predict significant shorter survival post-recurrence in a subgroup analysis, suggesting that this signature might be able to capture more aggressive tumors with high risk of recurrence (p<0.001, HR= 5.11, **Supplementary Figure 2**). We therefore explored whether combining these signatures could further improve our ability to predict outcome. At least one of these two signatures was present in 58 patients (44%), and having one or both signatures independently predicted both OS (HR=1.72, p=0.036) and recurrence (HR=3.16, p=0.001) **(Tables 4 and 5)**: patients with one or both signatures had 45% 5-year OS and 53% recurrence vs 67% 5-year OS and 19% recurrence when neither signature was present **(Figure 3C-D).** The presence of any of the two signatures also predicted recurrence when the analysis was limited to patients beyond Milan criteria based on preoperative imaging (n=94) (**Supplementary Figure 3, Supplementary Table 3**). Overall, 5-year recurrence rate was of 35.7% and was

significantly lower in patients CK19/S2 rule negative (20.8% vs 52.8%, p=0.003). Furthermore, the CK19/S2 rule, when applied to patients classified according to BCLC stage, significantly improved the prognostic prediction by stratifying patients in high and low risk for both recurrence and survival (**Supplementary Figures 4 and 5).**

Discussion

Almost 20 years after the seminal observation by Mazzaferro et al [1], we continue to rely on size and number of tumors for selecting patients with HCC for LT. Since then, the concept of a modest expansion beyond the Milan criteria has been proposed without gaining global acceptance [4, 38-39]. Most of the studies performed were retrospective, used pathological data and only included few patients with HCC beyond Milan criteria, thus leading to unrealistic good results because the outcome was “diluted” among a much larger pool of patients within Milan criteria. The largest series ever reported exploring expansion of Milan criteria led to the pathological up-to-7 rule where outcomes were significantly better for patients within this rule, as long as vascular invasion is not present [8]. After all, tumor size and number remain surrogates for the biological aggressiveness of HCC; henceforth more refined markers are needed.

While there have been multiple publications linking various genetic profiles of HCC with outcomes after hepatic resection, tumor biology in transplanted patients has not been assessed widely thus far. Most studies have relied on degree of HCC differentiation on biopsy, AFP and response to bridge therapy while on the waiting list [40-42]. Using allelic imbalance, we described that genomic instability of certain microsatellites selected for proximity to known oncogenes can help select patients beyond Milan criteria who are likely to have favorable outcomes after transplantation [43]. Because gene expression profiling could be eventually performed on needle liver biopsies in a pre-transplant setting [44], the positivity for each of the signatures can assist in the decision making of selecting patients for transplantation.

In our current study, we explored the tumor gene expression profiles for the presence of 22 previously reported prognostic signatures –reflecting HCC molecular

subclasses or activation of signaling pathways- in 132 patients transplanted with HCC beyond Milan criteria and key immunohistochemical staining and correlated the results with clinical variables and outcome. Sixteen signatures demonstrated association with recurrence and/or survival; among these the CK19 and the S2 signatures, both denoting progenitor cell features, were independent predictors of outcome when included in models incorporating clinical variables. As shown in **Figure 2**, both signatures cluster together along with others from hepatoblastoma, which overall reflect progenitor cell origin. In fact, CK19 is a molecular marker of progenitor cells, whereas S2 subclass [13] has been linked with high levels of expression of AFP even at early stages of the disease, and activation of mTOR and IGF signaling [21, 35]. We also found enrichment for Met and TGFb signaling in these patients. In our study, one and/or both signatures were present in 44% of cases and the overall 5 yr-survival and recurrence rate (53% and 45%, respectively) was discouraging. Conversely, in the 56% of patients harboring neither signature, OS was 67% and recurrence was 19% at 5 years, similar to the results of LT for HCC when conventional Milan criteria are applied. The capacity of our tool to rule out tumors with “poor biology” is directly reflected in the acceptable low recurrence rate.

In order to be used prospectively, the CK19/S2 gene signature needs to be validated when Milan criteria are defined on pre-operative imaging. Indeed, in the subgroup analysis of pre-operatively defined cases beyond Milan criteria, the CK19/S2 signature remained independently associated with recurrence. However, the signature no longer retained significance on multivariate analysis for overall survival, likely due to the smaller sample size. Overall, these findings support the possibility that genomic analysis of HCC performed prior to LT may enable expansion of the current imaging-

based selection criteria to include patients beyond Milan Criteria but with favorable genomic profiles, without significantly impacting clinical outcome.

Another interesting finding of our study was the relative genomic disparity observed in HCC cases treated with LT versus those undergoing resection. In this series of patients with HCC beyond Milan Criteria we observed a higher frequency of tumors demonstrating ≥ 6 signatures than we did in the study of patients undergoing liver resection [21].This may be explained, to some degree, by the more advanced clinical stage of cancers that received LT beyond Milan criteria versus those undergoing resection. While resection is limited to single tumors, nearly 95% of the patients in the current study were transplanted for multiple HCC.

We intend to validate these observations prospectively, but in order to establish the absence of the CK19 and S2 progenitor cell signatures as a basis for safe expansion of the indication for LT for patients with HCC, a number of barriers must be overcome. First, the study will have to be limited to patients identified pre-LT as being beyond Milan Criteria in order to be clinically relevant. The genomic heterogeneity of tumors is widely recognized, all the more so when the tumor is large or multinodular as in patients beyond Milan Criteria. While we have shown in a limited study that genomic heterogeneity in HCC rarely results in assignment of samples from the same tumor to different genomic classes [21], the degree to which a pre-LT biopsy accurately reflects the genomic landscape of the tumor remains unclear. Molecular heterogeneity between and within tumors can pose a risk for miss-classifications when selecting the area to biopsy prior LT. However, with the advancements of imaging techniques, a significant correlation has been shown between tumor enhancement and histological differentiation which could greatly assist in more accurate profiling [45].

On the other side, the impact on gene expression of pre-LT nonsurgical tumor treatment, that is commonly performed to down-stage HCC and forestalls progression while awaiting transplant, remains undefined. Furthermore, with long pre-LT waiting times before transplantation and the potential drop out of some patients, the stability of tumor gene expression profiles over time must be considered. Thus, the extent to which a biopsy done today reflects the nature of the tumor later on and the frequency with which biopsy will need to be repeated, need to be determined.

Despite its shortcomings, our observations provide initial insights into the understanding of tumor biology and clinical outcomes beyond size and number of HCC nodules. Those patients with tumors with “good biology” as defined by absence of signatures with progenitor cell traits (CK19/S2) represent half of the patients transplanted beyond Milan criteria in 2 institutions in the United States. Our seminal study provides the rationale for expanding the conventional criteria based upon genomic data, since outcomes are competitive with those achieved by HCC patients within Milan criteria. It is a novel, genomic, complementary tool to refine previously reported proposals, such as Up-to-7 rule [8]. Certainly, these results need to be validated using a separate external cohort of patients and then examined prospectively before they can be adopted by clinical guidelines.

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FIGURE LEGENDS

Figure 1: Flow chart of the study. The initial cohort included 770 patients that were consecutively transplanted for HCC at Mount Sinai Hospital, New York (n=590) and Mayo Clinic, Scottdale Arizona (n=180). A total of 206 patients were beyond Milan criteria based on the explant pathology. Tumors with HCC-ICC features, diffused pattern, necrotic tissue, macro-vascular invasion or metastasis were excluded. From the remaining 141 cases, 6 tumors with poor-quality RNA were discarded. Total RNA from 135 tumors was subjected to transcriptome profiling. Three samples had poor quality profile and were excluded and eventually 132 tumors were tested for the presence of previously reported outcome-associated gene signatures.

Figure 2: **Prediction of gene expression signatures.** Only signatures that were able to assign patients into good and poor prognosis groups with FDR>0.05 were included. **(A)** Each column represents different sample and each row different signature. Positivity of each signature is represented by red bars. Events (recurrence or 5-year death) are shown with black bars. **(B)** Visualization of cramer's V coefficient for the pair-wise comparison of gene signatures. The scale from blue to red represents the strength of correlation (red represents the highest correlation). The signatures are clustered according to their correlation.

Figure 3: **Kaplan-Meier curves and estimates of overall survival and recurrence in patients beyond Milan undergoing liver transplantation (n=132)**. Top figures show overall survival and recurrence in this cohort. Patients positive for either of CK19 or S2 signatures (progenitor-cell signatures) were assigned in a poor-outcome group.

Lower panels show overall survival and recurrence in patients beyond Milan with or without CK19/S2 signatures. P-values are calculated based on the log-rank test.

Title: Progenitor cell markers predict outcome of patients with Hepatocellular Carcinoma beyond Milan criteria undergoing liver transplantation

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List of abbreviations

HCC: Hepatocellular carcinoma

LT: Liver transplantation

CK19: Cytokeratin protein 19

HR: Hazard Ratio:

EpCAM: Epithelial cell adhesion molecule

OS: Overall survival

miVI: microvascular invasion

TGFb: Transforming growth factor beta

CT: Computed Tomography

MRI: Magnetic Resonance Imaging

AFP: Alpha-fetoprotein

IHC, immunohistochemical

RPS6: ribosomal protein S6

FDR: False discovery rate

H&E: Hematoxylin and eosin

PPV: Positive predictive value

NPV: Negative predictive value

HCV: Hepatitis C virus

HBV: Hepatitis B Virus

VIF: Variance Inflation Factor

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ABSTRACT

Background and Aims: In patients with hepatocellular carcinoma (HCC), liver transplantation (LT) is an excellent therapy if tumor characteristics are within the Milan criteria. We aimed to define genomic features enabling to identify HCC patients beyond Milan criteria who have acceptable transplant outcomes.

Methods: Among 770 consecutive HCC patients transplanted between 1990 and 2013, 132 had tumors exceeding Milan criteria on pathology and were enrolled in the study; 44% of the patients satisfied the 'up-to-7 rule' [7=sum of the size of the largest tumor and the number of tumors]. Explant tumors were assessed for genomic signatures and immunohistochemical markers associated with poor outcome.

Results: At a median follow-up of 88 months, 64 patients had died and 45 recurred; the 5-year overall survival (OS) and recurrence rates were 57% and 35%, respectively. Cytokeratin 19 (CK19) gene signature was independently associated with recurrence [Hazard ratio (HR)=2.95, p<0.001], along with tumor size (HR=3.37, p=0.023) and presence of satellites (HR=2.98, p=0.001). S2 subclass signature was independently associated with poor OS (HR=3.18, p=0.001), along with tumor size (HR=5.06, p<0.001) and up-to-7 rule (HR=2.50, p=0.002). Using the presence of progenitor cells markers (either CK19 or S2 signatures) patients were classified into poor-prognosis (n=58; 5-year recurrence 53%, survival 45%) and good-prognosis (n=74; 5-year recurrence 19%, survival 67%) (HR=3.16, p<0.001 for recurrence, and HR=1.72, p=0.04 for OS).

Conclusions:

HCC patients transplanted beyond Milan criteria without gene signatures of progenitor markers (CK19 and S2) achieved survival rates similar as those within Milan criteria.

Once prospectively validated, these markers may support a limited expansion of LT indications.

INTRODUCTION

Liver transplantation (LT) is an effective treatment option for hepatocellular carcinoma (HCC) when disease is defined by the widely accepted Milan criteria [1, 2]. Transplantation for patients within the Milan criteria generally yields a 5-year overall survival (OS) of 70% and a recurrence rate less than 15% [1-3]. Several efforts have been made to expand the criteria based on tumor size and number [4-8]. Although downstaging of tumors beyond Milan criteria is accepted by some UNOS regions, it has not yet been adopted by international consensus guidelines of LT [9] or guidelines of management of HCC [2]. Mazzaferro et al. proposed the up-to-7 rule [the sum of the number of tumor nodule(s) and the maximum diameter of the nodule(s) must not exceed the value of 7] which, in the absence of microvascular invasion (miVI) results in 5-year OS above 70% [8]. This study along with previously published data confirmed miVI to be a key predictor of recurrence in patients with HCC [10]. However, this information cannot be used in pre-transplant decision-making as an indication of transplantation since miVI is only diagnosed based on post-surgical histological assessment. This highlights the limitations of the current image-based prognostic algorithm for selecting HCC patients for LT.

Genome-wide transcriptome profiling has identified several key deregulated genes, molecular pathways and signatures associated with disease progression and prognosis in HCC [11, 12]. Activation of specific molecular pathways such as transforming growth factor-beta (TGFb) as well as presence of progenitor cell markers such as cytokeratin 19 (CK19) and epithelial cell adhesion molecule (EPCAM) have been associated with more aggressive biological tumor characteristics and rapid disease spread [13-35].

In a previous study, we identified gene signatures that significantly improved prediction of HCC recurrence after surgical resection [21]. Here we sought to define if gene signatures are also able to identify patients with HCC beyond Milan criteria who nevertheless may have acceptable outcomes with LT. In addition, we are providing the transcriptomic landscape of patients at more advanced states of the disease compared with those undergoing resection or transplantation according to Milan criteria, as per AASLD/EASL guidelines [2, 36].

MATERIALS AND METHODS

Patient cohorts and tissue samples

A total of 770 patients were transplanted for HCC between 1990 and 2013 at Mount Sinai Hospital, New York (n=590) and Mayo Clinic, Scottsdale-Arizona (n=180). Among these, we selected 132 patients (Mount Sinai: n=94, Mayo: n=38) with HCC beyond Milan criteria by pathological assessment, for whom base-line and follow-up clinical information and archived fixed tissue of viable tumor were available **(Figure 1).** The study protocol was approved by the respective Institutional Review Boards. See Supplementary Methods.

Genome-wide transcriptome profiling

Total RNA was subjected to transcriptome profiling using Whole-Genome DASL-HT (Illumina). See Supplementary Methods. GEO accession number: GSE62743.

Immunohistochemistry

Immunohistochemistry was performed for the evaluation of nuclear β-catenin as a surrogate for Wnt pathway activation; phosphorylation of ribosomal protein S6 (RPS6) as the major downstream effector of activated AKT-mTOR pathway; CK19 and EPCAM as surrogate of progenitor cell origin. See Supplementary Methods.

Bioinformatics and statistical analysis

Presence of previously reported HCC gene signatures (**Supplementary Table 1**) was evaluated (**Figure 2**). See Supplementary Methods.

RESULTS

Patient characteristics

Clinico-pathological data and outcomes of the patients are summarized in **Table 1**. Most of the patients were male (80%) and hepatitis C virus (HCV)-infected (71%). The median size of the largest tumor was 4 cm (range 1-9 cm). Tumors exceeded Milan criteria due to single tumor larger than 5 cm (5%), 2 to 3 tumors with the largest tumor larger than 3 cm (39%), or more than 3 tumors within the liver (57%). Satellite lesions (defined as HCC nodules < 2 cm in diameter within 2 cm from main tumor [37]) were present in 18% and miVI was present in 84% of the patients. Serum alpha fetoprotein (AFP) was > 200 mg/dL in 17% of cases. At the end of follow-up, 64 patients had died, OS at 5- and 10-years was 57% and 42%, respectively. HCC recurrence developed in 45 patients, 5 and 10-year cumulative recurrence rates were 35% and 44%, respectively (**Figure 3**).

Clinical variables identified as predictors of recurrence on multivariate analysis included tumor diameter >5 cm [p=0.023, Hazard Ratio (HR) = 3.37] and the presence of satellites (p<0.001, HR=2.98). Tumor diameter >5 cm (p<0.001, HR=5.06) and outside up-to-7 rule (HR=2.50, p=0.002) predicted poor survival (**Tables 2 and 3)**.

Prevalence of prognostic liver cancer gene signatures

Among the 22 liver cancer gene signatures tested, 16 were able to sort patients into good and poor prognosis groups with a FDR<0.05 (**Figure 2A**). The human [21] and rat [28] CK19 gene signatures were present in 40% and 42% of patients, respectively. Signatures associated with poor clinical outcome and/or more aggressive biological features were generally present in the same set of tumors. At least one poor-prognosis signature was present in 84 patients (64%), this being a higher rate than we have

previously reported in patients treated with resection [21]. Twenty-five tumors (19%) harbored more than 10 poor prognosis/aggressiveness gene signatures. VI signature was positive in 22% of cases, 93% of which were positive for histological miVI. The majority of tumors lacking poor prognosis/aggressive signatures were enriched by the S3 subclass signature (39/48, 81%), which is associated with smaller, higher proportion of well-differentiated tumors [13].

The gene signatures analyzed were consistent with 3 distinct clusters or major molecular classes of HCC **(Figure 2B)**: (1) *Proliferation and cell cycle activation* (Cluster A [16], Cholangiocarcinoma [29], Proliferation [20], Recurrence [24], S1 [13], TGFb [23], MET [18], and G3 [19] signatures), 2) *Proliferation-progenitor cell origin* (Hepatoblastoma C2 [26], Class S2 [13], EpCAM [25] and VI [22] signatures), which includes a subclass of CK*19* positive tumors (CK19 human [21] and rat [28] signatures), and 3) *non-proliferative class*, enriched by tumors positive for CTNNB1[20] or class S3 [13, 33]. Interestingly, there was no association between the underlying etiology [HCV, Hepatitis B Virus (HBV) or alcohol] and the expression of signatures.

Comparing the classification of signatures between this cohort and previously published results from resection cases [21], we noticed a higher frequency of tumors positive for $\geq$ 6 signatures of poor prognosis (32.6% vs 25%, respectively). Similarly, our cohort presented a higher prevalence of CK19 human signatures (41% vs 34%, respectively), suggesting that it includes more advanced tumors compared with our previous report on resected patients [21].

Immunohistochemical (IHC) analysis

CK19 immunostaining was positive in 6 tumors (5%), and was significantly associated with the presence of the human (p=0.03) and rat (p=0.04) CK19 gene signatures, as well as VI signature [22] (p<0.01) (**Supplementary Table 2**). EPCAM immunostaining was positive in 20 tumors (15%), and was significantly associated with EPCAM signature (p=0.01), human and rat CK19 signatures (p=0.01 and p=0.02, respectively), Proliferation subclass signature (p=0.01), and VI signature (p=0.01) (**Supplementary Table 2**). Nuclear staining of β-catenin was observed in 27 tumors (21%), and was positively associated with CTNNB1 class signature (p=0.01) (**Supplementary Figure 1, Supplementary Table 2**). In addition, 12 samples (9%) had overexpression of cytoplasmic β catenin with one third of these in CTNNB1 class (4/12, 33%). Phospho-RPS6 staining was positive in 75 tumors (57%) with no obvious correlation with any of the evaluated gene signatures.

Prognostic relevance of liver cancer gene signatures in LT treated patients beyond Milan

Prognostic gene signatures in HCC were initially developed in patients undergoing resection, a treatment indicated for early stages of the disease. Univariate Cox regression analysis demonstrated that VI [22], G3 subclass [19], and S2 subclass [13] signatures were associated with overall survival, along with patient's age, tumor size, and up-to-7 rule (**Table 2**). In the multivariate analysis only S2 signature [13] (HR=3.18, p=0.001), tumor diameter >5 cm (HR=5.06, p<0.001), and outside up-to-7 rule (HR=2.50, p=0.002) were independent predictors of survival. VI [22], G3 [19], Proliferation [20], CK19 human [21], CK19 rat [28], Cholangiocarcinoma [29], Cluster A [16] and S3 [13] gene signatures were associated with HCC recurrence in univariate

analysis along with tumor size, AFP and satellites from clinical variables as well as RPS6 from IHC (**Table 3**). In multivariate analysis, CK19 human [21] (HR=2.91, p=0.001), tumor diameter > 5 cm (HR=3.37, p=0.023) and satellites (HR=2.98, p<0.001) were independent predictors of recurrence.

Of note, histological miVI predicted neither survival nor recurrence, presumably because in this cohort it was present in the large majority of cases (112 patients, 84.8%). Also, IHC positivity for reported progenitor cell markers also failed to improve outcome prediction.

Combination of progenitor cell signatures (CK19 and S2) discriminates prognostic subgroups in transplant patients

Multivariate analyses demonstrated prognostic significance of two progenitor-related gene signatures, CK19 [21] and S2 [13]. Furthermore, S2 subclass signature was also able to predict significant shorter survival post-recurrence in a subgroup analysis, suggesting that this signature might be able to capture more aggressive tumors with high risk of recurrence (p<0.001, HR= 5.11, **Supplementary Figure 2**). We therefore explored whether combining these signatures could further improve our ability to predict outcome. At least one of these two signatures was present in 58 patients (44%), and having one or both signatures independently predicted both OS (HR=1.72, p=0.036) and recurrence (HR=3.16, p=0.001) **(Tables 4 and 5)**: patients with one or both signatures had 45% 5-year OS and 53% recurrence vs 67% 5-year OS and 19% recurrence when neither signature was present **(Figure 3C-D).** The presence of any of the two signatures also predicted recurrence when the analysis was limited to patients beyond Milan criteria based on preoperative imaging (n=94) (**Supplementary Figure 3, Supplementary Table 3**). Overall, 5-year recurrence rate was of 35.7% and was

significantly lower in patients CK19/S2 rule negative (20.8% vs 52.8%, p=0.003). Furthermore, the CK19/S2 rule, when applied to patients classified according to BCLC stage, significantly improved the prognostic prediction by stratifying patients in high and low risk for both recurrence and survival (**Supplementary Figures 4 and 5).**

Discussion

Almost 20 years after the seminal observation by Mazzaferro et al [1], we continue to rely on size and number of tumors for selecting patients with HCC for LT. Since then, the concept of a modest expansion beyond the Milan criteria has been proposed without gaining global acceptance [4, 38-39]. Most of the studies performed were retrospective, used pathological data and only included few patients with HCC beyond Milan criteria, thus leading to unrealistic good results because the outcome was “diluted” among a much larger pool of patients within Milan criteria. The largest series ever reported exploring expansion of Milan criteria led to the pathological up-to-7 rule where outcomes were significantly better for patients within this rule, as long as vascular invasion is not present [8]. After all, tumor size and number remain surrogates for the biological aggressiveness of HCC; henceforth more refined markers are needed.

While there have been multiple publications linking various genetic profiles of HCC with outcomes after hepatic resection, tumor biology in transplanted patients has not been assessed widely thus far. Most studies have relied on degree of HCC differentiation on biopsy, AFP and response to bridge therapy while on the waiting list [40-42]. Using allelic imbalance, we described that genomic instability of certain microsatellites selected for proximity to known oncogenes can help select patients beyond Milan criteria who are likely to have favorable outcomes after transplantation [43]. Because gene expression profiling could be eventually performed on needle liver biopsies in a pre-transplant setting [44], the positivity for each of the signatures can assist in the decision making of selecting patients for transplantation.

In our current study, we explored the tumor gene expression profiles for the presence of 22 previously reported prognostic signatures –reflecting HCC molecular

subclasses or activation of signaling pathways- in 132 patients transplanted with HCC beyond Milan criteria and key immunohistochemical staining and correlated the results with clinical variables and outcome. Sixteen signatures demonstrated association with recurrence and/or survival; among these the CK19 and the S2 signatures, both denoting progenitor cell features, were independent predictors of outcome when included in models incorporating clinical variables. As shown in **Figure 2**, both signatures cluster together along with others from hepatoblastoma, which overall reflect progenitor cell origin. In fact, CK19 is a molecular marker of progenitor cells, whereas S2 subclass [13] has been linked with high levels of expression of AFP even at early stages of the disease, and activation of mTOR and IGF signaling [21, 35]. We also found enrichment for Met and TGFb signaling in these patients. In our study, one and/or both signatures were present in 44% of cases and the overall 5 yr-survival and recurrence rate (53% and 45%, respectively) was discouraging. Conversely, in the 56% of patients harboring neither signature, OS was 67% and recurrence was 19% at 5 years, similar to the results of LT for HCC when conventional Milan criteria are applied. The capacity of our tool to rule out tumors with “poor biology” is directly reflected in the acceptable low recurrence rate.

In order to be used prospectively, the CK19/S2 gene signature needs to be validated when Milan criteria are defined on pre-operative imaging. Indeed, in the subgroup analysis of pre-operatively defined cases beyond Milan criteria, the CK19/S2 signature remained independently associated with recurrence. However, the signature no longer retained significance on multivariate analysis for overall survival, likely due to the smaller sample size. Overall, these findings support the possibility that genomic analysis of HCC performed prior to LT may enable expansion of the current imaging-

based selection criteria to include patients beyond Milan Criteria but with favorable genomic profiles, without significantly impacting clinical outcome.

Another interesting finding of our study was the relative genomic disparity observed in HCC cases treated with LT versus those undergoing resection. In this series of patients with HCC beyond Milan Criteria we observed a higher frequency of tumors demonstrating ≥ 6 signatures than we did in the study of patients undergoing liver resection [21].This may be explained, to some degree, by the more advanced clinical stage of cancers that received LT beyond Milan criteria versus those undergoing resection. While resection is limited to single tumors, nearly 95% of the patients in the current study were transplanted for multiple HCC.

We intend to validate these observations prospectively, but in order to establish the absence of the CK19 and S2 progenitor cell signatures as a basis for safe expansion of the indication for LT for patients with HCC, a number of barriers must be overcome. First, the study will have to be limited to patients identified pre-LT as being beyond Milan Criteria in order to be clinically relevant. The genomic heterogeneity of tumors is widely recognized, all the more so when the tumor is large or multinodular as in patients beyond Milan Criteria. While we have shown in a limited study that genomic heterogeneity in HCC rarely results in assignment of samples from the same tumor to different genomic classes [21], the degree to which a pre-LT biopsy accurately reflects the genomic landscape of the tumor remains unclear. Molecular heterogeneity between and within tumors can pose a risk for miss-classifications when selecting the area to biopsy prior LT. However, with the advancements of imaging techniques, a significant correlation has been shown between tumor enhancement and histological differentiation which could greatly assist in more accurate profiling [45].

On the other side, the impact on gene expression of pre-LT nonsurgical tumor treatment, that is commonly performed to down-stage HCC and forestalls progression while awaiting transplant, remains undefined. Furthermore, with long pre-LT waiting times before transplantation and the potential drop out of some patients, the stability of tumor gene expression profiles over time must be considered. Thus, the extent to which a biopsy done today reflects the nature of the tumor later on and the frequency with which biopsy will need to be repeated, need to be determined.

Despite its shortcomings, our observations provide initial insights into the understanding of tumor biology and clinical outcomes beyond size and number of HCC nodules. Those patients with tumors with “good biology” as defined by absence of signatures with progenitor cell traits (CK19/S2) represent half of the patients transplanted beyond Milan criteria in 2 institutions in the United States. Our seminal study provides the rationale for expanding the conventional criteria based upon genomic data, since outcomes are competitive with those achieved by HCC patients within Milan criteria. It is a novel, genomic, complementary tool to refine previously reported proposals, such as Up-to-7 rule [8]. Certainly, these results need to be validated using a separate external cohort of patients and then examined prospectively before they can be adopted by clinical guidelines.

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FIGURE LEGENDS

Figure 1: Flow chart of the study. The initial cohort included 770 patients that were consecutively transplanted for HCC at Mount Sinai Hospital, New York (n=590) and Mayo Clinic, Scottdale Arizona (n=180). A total of 206 patients were beyond Milan criteria based on the explant pathology. Tumors with HCC-ICC features, diffused pattern, necrotic tissue, macro-vascular invasion or metastasis were excluded. From the remaining 141 cases, 6 tumors with poor-quality RNA were discarded. Total RNA from 135 tumors was subjected to transcriptome profiling. Three samples had poor quality profile and were excluded and eventually 132 tumors were tested for the presence of previously reported outcome-associated gene signatures.

Figure 2: **Prediction of gene expression signatures.** Only signatures that were able to assign patients into good and poor prognosis groups with FDR>0.05 were included. **(A)** Each column represents different sample and each row different signature. Positivity of each signature is represented by red bars. Events (recurrence or 5-year death) are shown with black bars. **(B)** Visualization of cramer's V coefficient for the pair-wise comparison of gene signatures. The scale from blue to red represents the strength of correlation (red represents the highest correlation). The signatures are clustered according to their correlation.

Figure 3: **Kaplan-Meier curves and estimates of overall survival and recurrence in patients beyond Milan undergoing liver transplantation (n=132)**. Top figures show overall survival and recurrence in this cohort. Patients positive for either of CK19 or S2 signatures (progenitor-cell signatures) were assigned in a poor-outcome group.

Lower panels show overall survival and recurrence in patients beyond Milan with or without CK19/S2 signatures. P-values are calculated based on the log-rank test.

Tables

Table 1: Description of the main clinical and pathological characteristics of HCC patients undergoing LT beyond Milan criteria according to center and overall cohort (n=132)

	Overall cohort (n=132)	Mount Sinai (n=94)	Mayo Clinic (n=38)
Variable	**n (%)**	**n (%)**	**n (%)**
n	132 (100)	94 (71.2)	38 (28.8)
Age>60	39 (29.5)	28 (29.8)	11 (28.9)
Gender (males)	105 (79.5)	75 (79.8)	30 (78.9)
Transplant type			
Cadaveric donor	106 (80.3)	77 (81.9)	29 (76.3)
Living Donor	23 (17.4)	15 (16.0)	8 (21.1)
Underlying etiology*			
HCV	94 (71.2)	69 (73.4)	25 (65.8)
HBV	19 (14.4)	17 (18.1)	2 (5.26)
Alcohol	24 (18.2)	15 (16.0)	9 (23.7)
Other	10 (7.6)	8 (6.4)	4 (10.5)
Larger tumor size			
≤3cm	37 (28.0)	27 (28.7)	10 (26.3)
3-5cm	63 (47.7)	45 (47.9)	18 (47.4)
≥5cm	32 (24.2)	22 (23.4)	10 (26.3)
Number of tumors			
1-2	39 (29.5)	25 (26.6)	14 (36.8)
3-4	40 (30.3)	29 (30.8)	11 (28.9)
>5	53 (40.2)	40 (42.6)	13 (34.2)
Reason for extended criteria			
single tumor >5cm tumor	6 (4.5)	4 (4.3)	2 (5.3)
2-3 tumors, larger >3cm	51 (38.6)	34 (36.2)	17(44.7)
>3 tumors	74 (56.1)	55 (58.5)	19 (50)
Pathological markers			
Well Differentiated	15 (11.4)	8 (8.5)	7 (18.4)
Moderately Differentiated	52 (39.4)	32 (34.0)	20 (52.6)
Poorly Differentiated	65 (49.2)	54 (57.4)	11 (28.9)
Satellites	24 (18.2)	22 (23.4)	2 (5.26)
Microvascular invasion	112 (84.8)	90 (95.7)	22 (57.9)
Satisfying up-to-7 rule	56 (44.1)	37 (41.1)	19 (51.4)
Within Mil pre-op	33 (26)	11 (12.4)	22 (57.9)
Alpha-fetoprotein >100mg/dL	30 (22.7)	25 (26.6)	5 (13.2)
Alpha-fetoprotein >200mg/dL	23 (17.4)	19 (20.2)	4 (10.5)
Albumin (<3.5mg/dL)	87 (65.9)	68 (72.3)	19(50)
Bilirubin (>1mg/dL)	100 (75.8)	77 (81.9)	23 (60.5)
Child Pugh			
Class A	53 (40.2)	29 (36.3)	24 (63.2)
Class B	36 (30.5)	28 (35.0)	8 (21)
Class C	29 (24.6)	23 (28.8)	6 (15.8)
BCLC*			
A	27 (23.9)	23 (29.9)	4 (11.1)
B	86 (76.1)	54 (70.1)	32 (88.9)
Events			
Deaths	64 (48.5)	51 (54.3)	13 (34.2)
Overall Recurrence	45 (34.1)	34 (36.2)	11 (28.9)
Early Recurrence (<2years)	30 (22.7)	24 (25.5)	6 (15.8)
Follow up (months) median[Q25-Q75]	88 (65-122)	98 (78-138)	66 (37-80)
5-year Overall survival (%)l	57.0	54.5	64.7
5-year Recurrence (%)	34.5	35.7	31.7

*BCLC defined with pre-operative clinical and radiological variables. Because patients were treated with liver transplantation, Child C patients were not considered BCLC D and were relocated regardless of liver function.

Table 2: Univariate and Multivariate analysis of clinical, pathological and gene signatures for overall survival in patients beyond Milan criteria (n=132)

		Patients		Overall Survival, events n=64					
				Univariate analysis			Multivariate analysis		
Gene expression signatures		**Pos**	**Rest**	**pval**	**HR**	**95% CI**	**pval**	**HR**	**95% CI**
VI signature [22]		29	103	**0.040**	**1.760**	**1.02-3.04**			
G3 signature [19]		40	92	**0.040**	**1.705**	**1.02-2.86**			
Proliferation [20]		39	93	0.250	1.356	0.80-2.29			
CK19 human [21]		53	79	0.054	1.611	0.99-2.63			
CK19 rat [28]		56	76	0.130	1.457	0.89-2.38			
MET signature [18]		34	98	0.458	0.800	0.44-1.45			
Hepatoblastoma_C2 [26]		6	126	0.086	2.369	0.86-6.56			
CTNNB1 [20]		30	102	0.208	1.435	0.81-2.53			
EpCAM [25]		26	106	0.411	1.281	0.71-2.32			
Cholangiocarcinoma [29]		44	88	0.471	1.209	0.72-2.03			
Recurrence [24]		41	91	0.713	1.104	0.65-1.88			
Cluster A [16]		45	87	0.369	1.264	0.76-2.11			
TGFb [23]		38	94	0.762	0.918	0.53-1.60			
S1 [13]		42	90	0.545	0.845	0.49-1.46			
S2 [13]		15	117	**0.011**	**2.274**	**1.18-4.37**	**0.001**	**3.177**	**1.60-6.31**
S3 [13]		47	85	0.358	0.781	0.50-1.33			
IHC									
CK19		6	126	0.571	1.396	0.44-4.46			
EpCAM		20	112	0.096	0.497	0.21-1.15			
bcatenin		27	105	0.334	1.329	0.74-2.37			
RPS6		75	57	0.173	1.414	0.86-2.33			
Clinical Variables									
Male gender		105	27	0.441	0.775	0.40-1.49			
Age (>60)		39	93	**0.031**	**1.730**	1.04-2.87			
Etiology	HCV	94	38	0.168	1.512	0.84-2.74			
	HBV	19	113	0.381	0.718	0.34-1.51			
	Alcohol	24	108	0.363	1.316	0.73-2.39			
Single tumor >5cm		6	126	**<0.001**	**4.289**	**1.83-10.07**	**<0.001**	**5.057**	**2.06-12.44**
2-3 tumors, largest >3cm		51	81	0.118	0.659	0.39-1.12			
No of tumors (>3)		75	57	0.675	1.112	0.68-1.83			
AFP (>200mg/dL)		23	109	0.087	1.650	0.92-2.95			
MiVI		112	20	0.051	2.631	0.96-7.24			
Satellites		24	108	0.056	1.724	0.98-3.04			
Differentiation (Poor)		65	67	0.183	1.398	0.85-2.30			
Outside Up-to-7		69	56	**0.018**	**1.869**	**1.10-3.17**	**0.002**	**2.499**	**1.42-4.42**
Within Milan in pre-op assessment		32	95	0.531	0.818	0.44-1.54			
BCLC A		27	86	0.184	1.491	0.82-2.70			
Child A		53	65	0.322	0.758	0.44-1.32			

Table 3: Univariate and Multivariate analysis of clinical, pathological and gene signatures for overall recurrence in patients beyond Milan criteria (n=132)

		Patients		Overall Recurrence, events n=45					
				Univariate analysis			Multivariate analysis		
Gene expression signatures		**Pos**	**Rest**	**pval**	**HR**	**95% CI**	**pval**	**HR**	**95% CI**
VI signature [22]		29	103	**0.008**	**2.262**	**1.22-4.21**			
G3 signature [19]		40	92	**0.038**	**1.861**	**1.02-3.39**			
Proliferation [20]		39	93	**0.004**	**2.297**	**1.28-4.14**			
CK19 human [21]		53	79	**<0.001**	**3.031**	**1.66-5.54**	**0.001**	**2.905**	**1.59-5.32**
CK19 rat [28]		56	76	**0.001**	**2.676**	**1.46-4.90**			
MET signature [18]		34	98	0.181	1.520	0.82-2.83			
Hepatoblastoma_C2 [26]		6	126	0.702	1.318	0.32-5.47			
CTNNB1 [20]		30	102	0.986	0.994	0.48-2.07			
EpCAM [25]		26	106	0.461	1.301	0.64-2.63			
Cholangiocarcinoma [29]		44	88	**0.036**	**1.855**	**1.03-3.34**			
Recurrence [24]		41	91	0.366	1.323	0.72-2.44			
Cluster A [16]		45	87	**0.046**	**1.802**	**1.00-3.25**			
TGFb [23]		38	94	0.917	1.035	0.54-1.97			
S1 [13]		42	90	0.529	1.215	0.66-2.24			
S2 [13]		15	117	0.132	1.842	0.82-4.14			
S3 [13]		47	85	**0.012**	**0.408**	**0.20-0.85**			
IHC									
CK19		6	126	0.321	1.610	0.38-6.85			
EpCAM		20	112	0.416	0.702	0.30-1.66			
bcatenin		27	105	0.381	1.353	0.68-2.68			
RPS6		75	57	**0.025**	**2.024**	**1.07-3.82**			
Clinical Variables									
Male gender		105	27	0.558	0.797	0.37-1.71			
Age (>60)		39	93	0.782	1.095	0.57-2.09			
Etiology	HCV	94	38	0.254	1.499	0.74-3.03			
	HBV	19	113	0.204	0.521	0.19-1.46			
	Alcohol	24	108	0.679	1.170	0.56-2.42			
Single tumor >5cm		6	126	**0.016**	**3.306**	**1.17-9.34**	**0.023**	**3.374**	**1.18-9.63**
2-3 tumors, largest >3cm		51	81	0.168	0.644	0.34-1.21			
No of tumors (>3)		75	57	0.580	1.183	0.65-2.15			
AFP (>200mg/dL)		23	109	**0.003**	**2.579**	**1.35-4.93**			
MiVI		112	20	0.181	1.982	0.71-5.54			
Satellites		24	108	**<0.001**	**3.069**	**1.65-5.72**	**<0.001**	**2.978**	**1.60-5.55**
Differentiation (Poor)		65	67	0.271	1.388	0.77-2.50			
Outside Up-to-7		69	56	0.241	1.444	0.78-2.67			
Within Milan in pre-op assessment		32	95	0.382	0.710	0.33-1.53			
BCLC A		27	86	0.962	1.018	0.48-2.15			
Child A		53	65	0.587	1.187	0.64-2.12			

Table 4: Univariate and Multivariate analysis of CK19/S2 score for overall survival in patients beyond Milan (n=132)

		Patients		Overall Survival, events n=64					
				Univariate analysis			Multivariate analysis		
Gene expression Signatures		**Pos**	**Rest**	**pval**	**HR**	**95% CI**	**Pval**	**HR**	**95% CI**
CK19 [21] and/or S2 pos [13]		58	74	**0.030**	**1.17**	**1.04-2.79**	**0.036**	**1.719**	**1.034-2.85**
IHC									
CK19		6	126	0.571	1.396	0.44-4.46			
EpCAM		20	112	0.096	0.497	0.21-1.15			
bcatenin		27	105	0.334	1.329	0.74-2.37			
RPS6		75	57	0.173	1.414	0.86-2.33			
Clinical Variables									
Male gender		105	27	0.441	0.775	0.40-1.49			
Age (>60)		39	93	**0.031**	**1.730**	1.04-2.87			
Etiology	HCV	94	38	0.168	1.512	0.84-2.74			
	HBV	19	113	0.381	0.718	0.34-1.51			
	Alcohol	24	108	0.363	1.316	0.73-2.39			
Single tumor >5cm		6	126	**<0.001**	**4.289**	**1.83-10.07**	**<0.001**	**5.510**	**2.26-13.45**
Two-Three tumors, larger >3cm		51	81	0.118	0.659	0.39-1.12			
No of tumors (>3)		75	57	0.675	1.112	0.68-1.83			
AFP (>200mg/dL)		23	109	0.087	1.650	0.92-2.95			
MiVI		112	20	0.051	2.631	0.96-7.24			
Satellites		24	108	0.056	1.724	0.98-3.04			
Differentiation (Poor)		65	67	0.183	1.398	0.85-2.30			
Outside Up-to-7		69	56	**0.018**	**1.869**	**1.10-3.17**	**0.007**	**2.113**	**1.23-3.64**
Within Milan in pre-op assessment		32	95	0.531	0.818	0.44-1.54			
BCLC A		27	86	0.184	1.491	0.82-2.70			
Child A		53	65	0.322	0.758	0.44-1.32			

Table 5: Univariate and Multivariate analysis of CK19/S2 score for overall recurrence in patients beyond Milan (n=132)

Gene expression Signatures		Patients Pos	Patients Rest	Overall Recurrence, events n=45					
				Univariate analysis			Multivariate analysis		
				pval	HR	95% CI	pval	HR	95% CI
CK19 [21] and/or S2 pos [13]		58	74	**<0.001**	**3.192**	**1.67-5.60**	**<0.001**	**3.162**	**1.70-5.89**
IHC									
CK19		6	126	0.321	1.610	0.38-6.85			
EpCAM		20	112	0.416	0.702	0.30-1.66			
bcatenin		27	105	0.381	1.353	0.68-2.68			
RPS6		75	57	**0.025**	**2.024**	**1.07-3.82**			
Clinical Variables									
Male gender		105	27	0.558	0.797	0.37-1.71			
Age (>60)		39	93	0.782	1.095	0.57-2.09			
Etiology	HCV	94	38	0.254	1.499	0.74-3.03			
	HBV	19	113	0.204	0.521	0.19-1.46			
	Alcohol	24	108	0.679	1.170	0.56-2.42			
Single tumor >5cm		6	126	**0.016**	**3.306**	**1.17-9.34**	**0.019**	**3.520**	**1.23-10.06**
Two-Three tumors, larger >3cm		51	81	0.168	0.644	0.34-1.21			
No of tumors (>3)		75	57	0.580	1.183	0.65-2.15			
AFP (>200mg/dL)		23	109	**0.003**	**2.579**	**1.35-4.93**			
MiVI		112	20	0.181	1.982	0.71-5.54			
Satellites		24	108	**<0.001**	**3.069**	**1.65-5.72**	**<0.001**	**3.078**	**1.65-5.74**
Differentiation (Poor)		65	67	0.271	1.388	0.77-2.50			
Outside Up-to-7		69	56	0.241	1.444	0.78-2.67			
Within Milan in pre-op assessment		32	95	0.382	0.710	0.33-1.53			
BCLC A		27	86	0.962	1.018	0.48-2.15			
Child A		53	65	0.587	1.187	0.64-2.12			

Fig. 1

Figure 1

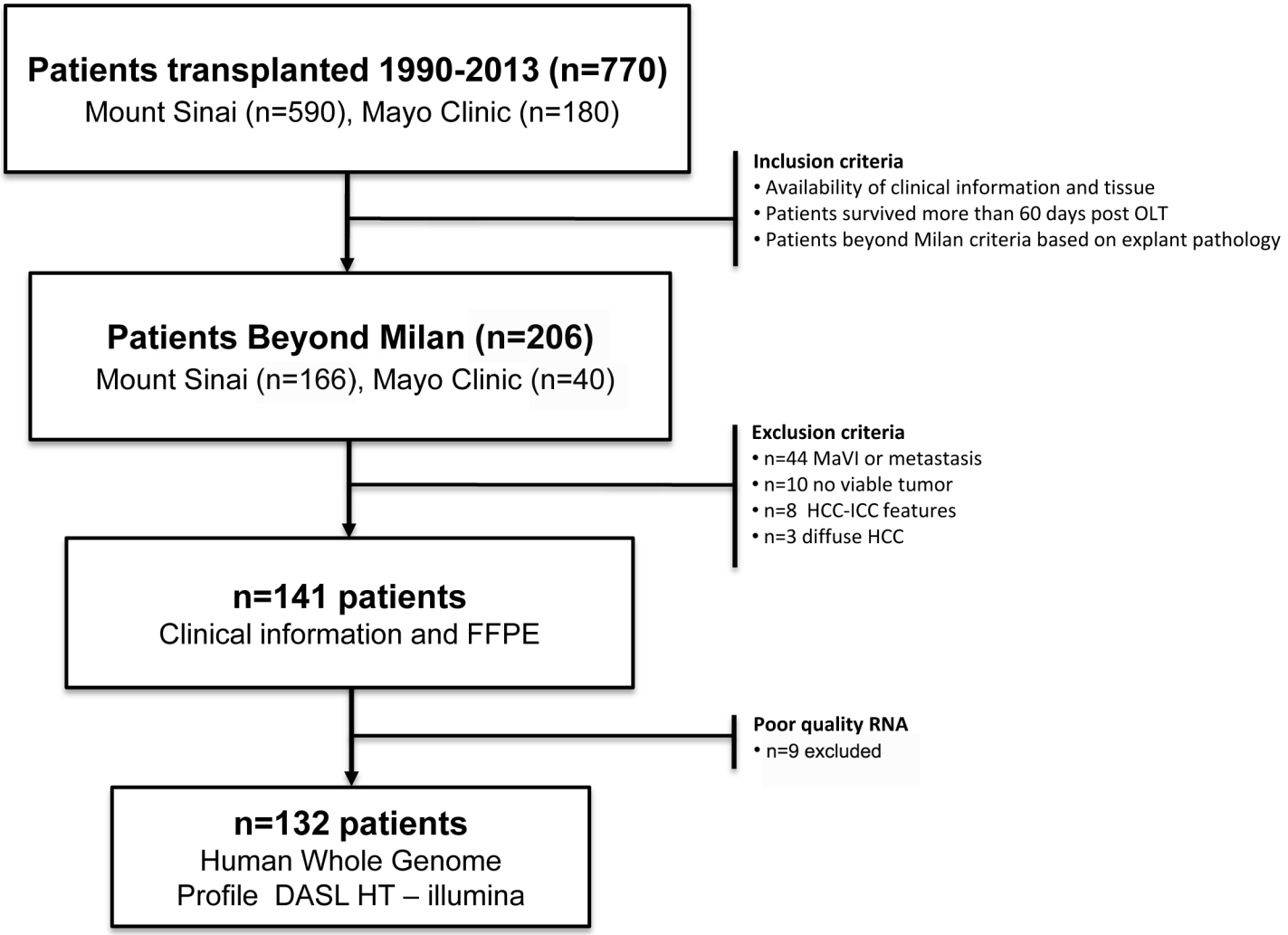
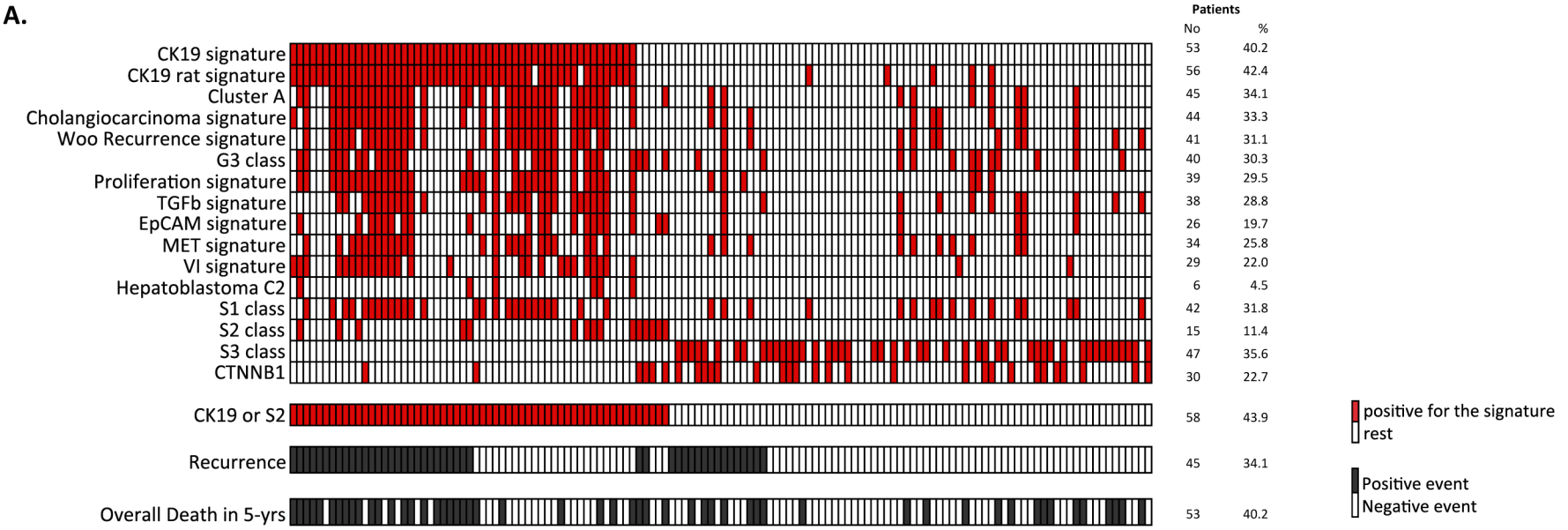


Fig. 2

Figure 2

A.



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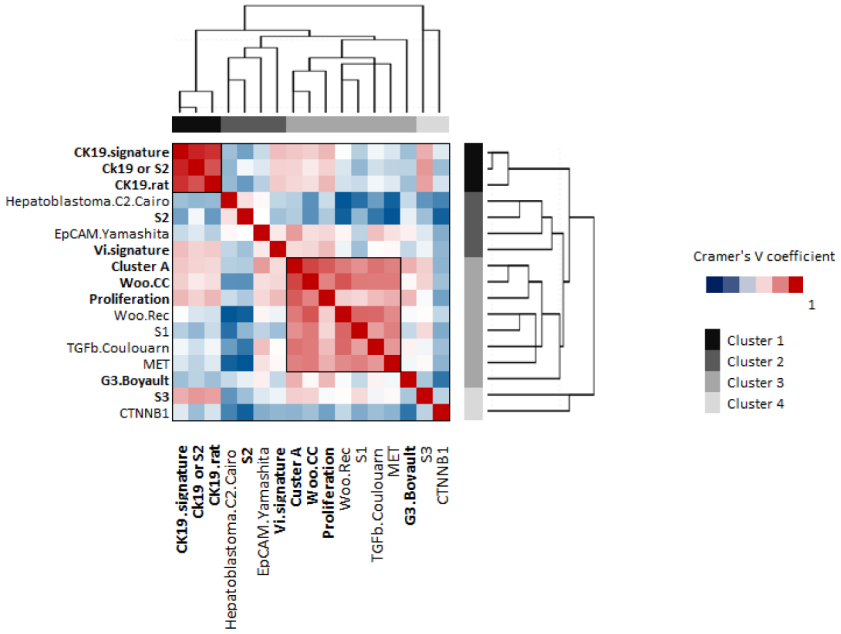
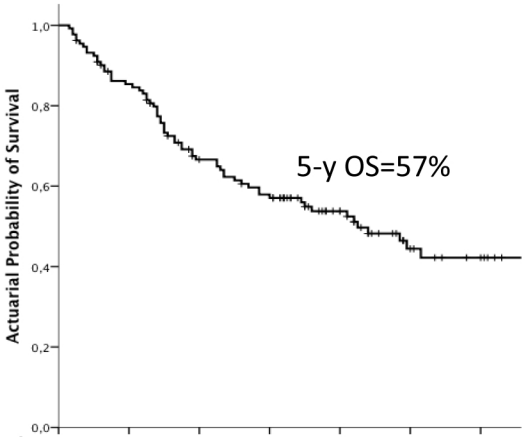


Fig. 3

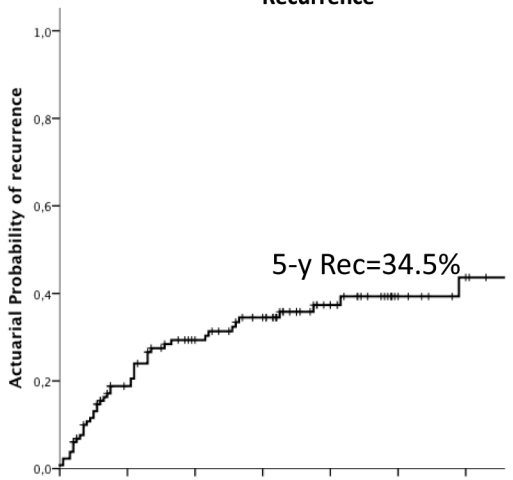
Figure 3

A.



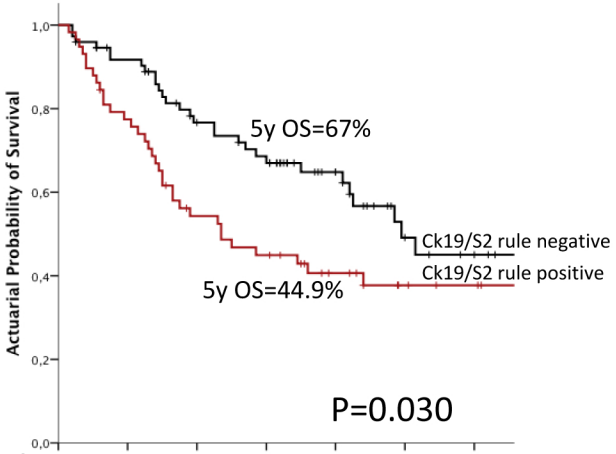
months	0	20	40	60	80	100	120
Pts at risk	132	108	77	65	41	21	15

B.



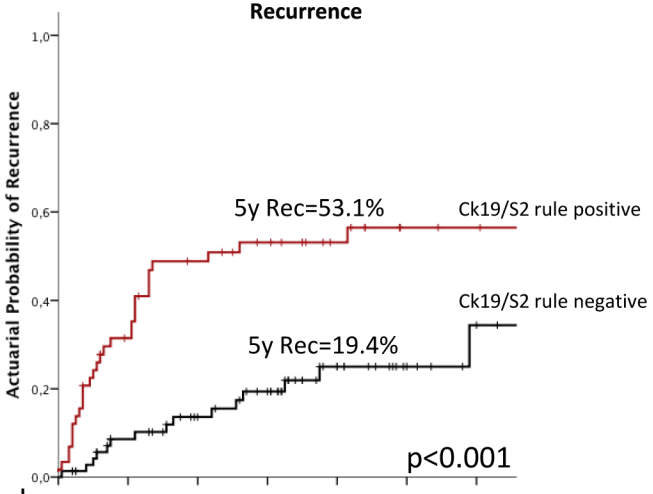
months	0	20	40	60	80	100	120
Pts at risk	132	94	71	58	34	18	12

C.



months	0	20	40	60	80	100	120
CK19/S2 (+)	58	44	29	24	16	8	7
Rest	74	64	52	41	25	12	8

D.



months	0	20	40	60	80	100	120
CK19/S2 (+)	58	39	25	20	14	7	6
Rest	74	58	46	38	20	11	6

Supplementary material

Click here to download Supplementary material: Supplementary_Miltiadous_Miltiadous_Llovet_July2015.docx